A Short−Form Paper for Antimicrobial Agents and Chemotherapy

Title: The novel MFS efflux pump SxtP, regulated by the LysR−Type transcriptional activator SxtR, is involved in the susceptibility to sulfamethoxazole/trimethoprim (SXT) and the pathogenesis of *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii is a notorious opportunistic pathogen responsible for healthcare−associated infections worldwide. Efflux pumps play crucial roles in mediating antimicrobial resistance, motility and virulence. In this study, we present the identification and characterization of the new *A. baumannii* efflux pump SxtP belonging to the MFS superfamily (Major Facilitator Superfamily), along with its associated activator LTTR (LysR−Type Transcriptional Regulator) SxtR, demonstrating their roles in sulfamethoxazole/trimethoprim (also known as co−trimoxazole or SXT) resistance, surface−associated motility and virulence.

Acinetobacter baumannii is a Gram−negative bacterium that has become a significant threat in hospital settings due to its ability to acquire and rapidly develop resistance to antimicrobial agents (1). One of the primary mechanisms underlying its multidrug resistance is the expression of efflux pumps that extrude antimicrobial agents from the internal environment of the bacterial cell. Multidrug efflux pumps are frequently encoded adjacent to a divergently transcribed regulatory protein that controls the expression of the pump gene in response to the transported substrates. For example, AdeL is an LTTR (LysR−Type Transcriptional Regulator) which is encoded opposite to the *adeFGH* operon and represses these genes encoding an RND (Resistance Nodulation Division) efflux system in *A. baumannii* (2). Another example of LTTR in *A. baumannii* is AceR, which controls the expression, in this case as an activator, of the divergently encoded PACE (Proteobacterial Antimicrobial Compound Efflux) superfamily efflux pump AceI (3). LTTRs are the largest family of prokaryotic transcriptional regulators and can function either as activators or repressors, controlling gene products involved across diverse processes, including antimicrobial resistance, motility, and virulence (2–5).

By analyzing the genome of the *A. baumannii* ATCC 17978 strain (GenBank: CP018664.1), we have identified the genes *AUO97_05400* (protein_id="APP30282.1") and *AUO97_05405* (protein_id="APP30283.1"), which are located adjacent to each other and oriented in opposite directions. The *AUO97_05400* gene is located upstream of *AUO97_05405* and they are separated by a short intergenic region of 138 base pairs (bp). The *AUO97_05400* gene is 921 bp in length and encodes a putative transcriptional regulator (LTTR−type). According to the deduced amino acid sequence, the putative LTTR protein consists of 306 residues and has a molecular mass of 33.65 kDa. The HTH (Helix−Turn−Helix) program (https://npsa−prabi.ibcp.fr) predicted the presence of this HTH DNA−binding motif typical of the LTTR family between residues 7 and 28, specifically KNFTKAAQRLNMSQPPLSMQIR (score 5.13, probability 100 %). The *AUO97_05405* gene is 1,245 bp in length and encodes a putative efflux pump (MFS type). Beyond the inferred sequence of amino acids, the putative MFS protein consists of 414

residues and has a molecular mass of 44.4 kDa. Based on predictions of its secondary structure and transmembrane topology using the Protter program (https://wlab.ethz.ch/protter/), this putative MFS is composed of 12 α−helical transmembrane segments, with both the N− and C−termini located in the cytoplasm (data not shown). An *in−silico* search of the sequences of the *A. baumannii* deposited in the NCBI database was carried out to determine the presence of the identified genes in the genomes of other strains within this species. This search involved 1,388 *A. baumannii* complete chromosome sequences and the presence of both genes was detected in 94.3 % of the analyzed genomes, indicating a strong conservation of these genes in *A. baumannii* strains (see details in supplementary material).

To determine whether the lack of the genes encoding the LTTR and MFS proteins alter antimicrobial susceptibilities, an internal ~500 bp of each gene amplified by PCR was cloned into the suicide vector pCR−BluntII−TOPO (Invitrogen) to disrupt the corresponding ORFs of *A. baumannii* target strains (ATCC 17978 and a clinical isolate) as previously described (6). All oligonucleotides used in this work are listed in Table S1. Antimicrobial susceptibility was determined by both disk diffusion and broth microdilution assays, conducted in accordance with the Clinical & Laboratory Standards Institute (CLSI) guidelines . Compared to the wild−type (WT) parental strain, both *A. baumannii* knockouts strains exhibited a 256−fold increase in susceptibility to sulfamethoxazole and a 128−fold increase in susceptibility to co−trimoxazole (a combination of trimethoprim and sulfamethoxazole in a 1:5 ratio). Additionally, the knockout strains shown 2− to 4−fold higher susceptibilities to the tested aminoglycosides (amikacin, gentamicin and apramycin), tetracyclines (minocycline and tetracycline), quinolones (levofloxacin, enrofloxacin and nalidixic acid) as well as to rifampin and trimethoprim (Table 1). In contrast, the mutants and their WT parental strain did not differ in their susceptibilities to the tested β−lactams (ceftazidime, cefotaxime, ticarcillin), macrolides (erythromycin), polymyxins (colistin) or to other antimicrobials such as chloramphenicol or ciprofloxacin (Table 1). Due to the high susceptibility of both mutants to co-trimoxazole, also known as SXT, we

renamed the genes *AUO97_05405* and *AUO97_05400* to *sxtP* (Pump) and *sxtR* (Regulator), respectively. As both the transcriptional regulator and efflux pump knockouts show similar antimicrobial profiles, it can be hypothesized that the LTTR protein SxtR could be a direct regulator of the MFS efflux pump SxtP, potentially playing a role as an activator. To further corroborate this inference, EMSAs (Electrophoretic Mobility Shift Assays) and RT−qPCRs were carried out as previously reported (7). Results obtained show that the SxtR purified protein specifically recognizes and binds the 138−bp intergenic region between the genes coding for the SxtR and SxtP proteins (Fig. 1), which includes a TTA−N_7−TAA motif (specifically: TTA−AATAGCT−TAA), typical of an LTTR box which is implicated in DNA binding by LTTRs (4). Accordingly, the expression level of the SxtP encoding gene was observed to be decreased by 2 to 4−fold in three independent RT−qPCR analyses conducted on the *sxtR* defective mutant compared to the ATCC 17978 WT strain and similar results were obtained with an *A. baumannii sxtR* knockout constructed from the clinical strain UAB_M1B07, which was recently isolated from a hospital in the metropolitan area of Barcelona, Spain. It is worth noting that although this strain was more sensitive to co−trimoxazole (MIC 0.125 mg/L) than the ATCC 17978 strain (see Table 1), the inactivation of the *sxtR* gene in the UAB_M1B07 strain reduced the MIC to 0.032 mg/L. All together, these results demonstrate that the SxtR protein acts as a direct transcriptional activator of the gene encoding the SxtP efflux pump.

Additionally, to assess the inducibility of this regulatory system, RT-qPCR experiments were performed using RNA extracted from cultures treated in the exponential growth phase with different antimicrobials (sulfamethoxazole, levofloxacin, or amikacin) at various subinhibitory concentrations for two hours. The results obtained showed no significant changes in the expression of the system with any of the tested antimicrobials compared to untreated cultures (data not shown). It is important to note that the presence of expelled antimicrobials does not necessarily indicate that they are inducers of these systems, as previously demonstrated. For example, the *Pseudomonas aeruginosa* MexAB-OprM efflux pump, which extrudes several

classes of antimicrobials including chloramphenicol, does not exhibit increased expression in the presence of this compound (8).

Efflux pumps may play a crucial role in bacterial pathogenicity by transporting antimicrobial molecules, secreting surfactants for surface−associated motility, and expelling virulence factors (9, 10). In this context, the identified genes were investigated for their potential roles in surface−associated motility and virulence of *A. baumannii*. Following priorly described methods (11), motility assays on 0.5 % agar plates exhibited a noteworthy decrease in motility for both *sxtP* and *sxtR* knockouts (Fig. 2A). It is worth noting that growth curves in liquid Luria−Bertani (LB) medium remained unaltered, this rules out changes in growth rate as the underlying cause for the observed motility impairments (data not shown). Moreover, assays in the *Galleria mellonella* animal model exhibited a significant reduction in virulence upon the inactivation of either SxtR− or SxtP−encoding genes (Fig. 2B), emphasizing their importance in the pathogenicity of *A. baumannii*.

Data reported here demonstrate that the newly characterized MFS efflux pump SxtP, along with its transcriptional activator SxtR, are widely distributed among clinical *A. baumannii* isolates and play an important role not only in antimicrobial resistance, specially to co−trimoxazole, but also in surface−associated motility, and virulence in *A. baumannii*. These findings underscore the potential of these proteins, among other efflux pumps and their transcriptional regulators, as putative new targets for developing novel therapeutic strategies against this nosocomial pathogen.

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Table 1. Minimum inhibitory concentrations (MICs, mg/liter) of the indicated antimicrobials for wild–type *A. baumannii* ATCC 17978 (WT) and the derivative mutants lacking the genes encoding either the LysR or the MFS proteins.

	WT	SxtP$^-$	SxtR$^-$
Sulfamethoxazole	**256**	**1**	**1**
Co–trimoxazole	**4**	**0.0031**	**0.0031**
Trimethoprim	**16**	**8**	**8**
Amikacin	**2**	**0.5**	**1**
Gentamicin	**1**	**0.5**	**0.5**
Apramycin	**4**	**2**	**2**
Minocycline	**0.125**	**0.062**	**0.062**
Tetracycline	**1**	**0.5**	**0.5**
Tigecycline	**0.25**	**0.25**	**0.25**
Levofloxacin	**0.031**	**0.016**	**0.016**
Enrofloxacin	**0.0062**	**0.0031**	**0.0031**
Nalidixic acid	**8**	**4**	**4**
Rifampin	**4**	**2**	**2**
Ceftazidime	**4**	**4**	**4**
Cefotaxime	**8**	**8**	**8**
Ticarcillin	**32**	**32**	**32**
Erythromycin	**8**	**8**	**8**
Colistin	**0.5**	**0.5**	**0.5**
Chloramphenicol	**32**	**32**	**32**
Ciprofloxacin	**0.25**	**0.25**	**0.25**

FIGURE LEGENDS

Fig. 1. (A) Intergenic region showing the 138 bp between the *sxtP* and *sxtR* genes in *A. baumannii* strain ATCC 17978. Surrounding genes are also shown. The TTA−N_7−TAA DNA motif is shown in bold and underlined. **(B)** EMSA of a DIG−labeled DNA fragment containing the intergenic sequence present between the genes coding SxtP and SxtR proteins. The experiment was carried out in the presence (+) or absence (−) of 2 μM of SxtR protein, with at least a 10−fold molar excess of non−labeled and non−specific DNA in all lanes, and non−labeled and specific DNA (third lane). All EMSAs were performed at least three times obtaining reproducible results. A representative image is shown.

Fig. 2. **(A)** Surface−associated motility assays of wild−type *A. baumannii* strain ATCC 17978 (WT), and the derivative mutants lacking the genes encoding either the SxtP or the SxtR proteins. All surface−associated motility assays were performed at least three times obtaining reproducible results. A representative image is shown. **(B)** *G. mellonella* killing assay of the specified strains. Larvae (n = 10 per group) were inoculated with either ~10^6 CFU of the indicated strain or PBS as a negative control (not shown). *P < 0.001 compared to the *A. baumannii* parental ATCC 17978 strain (WT). All *G. mellonella* killing experiments were performed at least three times obtaining reproducible results. A representative graph of larval survival is shown.

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